

1        Recombinant HcGAPDH Protein Expressed on Probiotic *Bacillus*  
2        *subtilis* Spores Protects Sheep from *Haemonchus contortus* Infection  
3        by Inducing both Humoral and Cell-mediated Responses

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18        (AD)

## 19 **Abstract**

20 Probiotic Bacillales have been shown effective in controlling pathogens. In  
21 particular, live probiotic bacteria are thought to improve the composition of gastrointestinal  
22 microbiota, and to reduce pathogen colonization. However, how probiotics regulate immune  
23 responses and protect the host from parasitic infection remains largely unknown. In this  
24 study, we investigated whether Bacillales can be used against *Haemonchus contortus*, a  
25 parasitic nematode that infects small ruminants in sheep and goats worldwide. Using 16S  
26 ribosomal RNA sequencing, we found that Bacillales was highly depleted in the abomasal  
27 microbiota of sheep infected with *H. contortus*. We constructed a recombinant *Bacillus*  
28 *subtilis* strain (rBS<sup>CotB-HcG</sup>) that express glyceraldehyde-3-phosphate dehydrogenase of *H.*  
29 *contortus* (HcGAPDH) on its spore surface. However, mice orally administrated with the  
30 rBS<sup>CotB-HcG</sup> strain showed strong Th1-dominated immune responses; and sheep  
31 administrated *per os* with rBS<sup>CotB-HcG</sup> showed increased proliferation of peripheral blood  
32 mononuclear cells, elevated anti-HcGAPDH IgG levels in sera, and higher anti-HcGAPDH  
33 sIgA levels in intestinal mucus. In addition, treatment of *H. contortus* infected sheep with  
34 rBS<sup>CotB-HcG</sup> (Hc+rBS<sup>CotB-HcG</sup>) promoted the abundance of probiotic species in the abomasal  
35 microbiota; it also improved the average weight gain of the sheep by 27.7%. These  
36 Hc+rBS<sup>CotB-HcG</sup> sheep have reduced number of eggs per gram of feces (by 84.1%) and  
37 worm burdens (by 71.5%), with alleviated abomasal damage by *H. contortus*. Collectively,  
38 our data demonstrate the protective roles of CotB-HcGAPDH-expressing *B. subtilis* spores  
39 against *H. contortus* infection, suggesting a potential value of using this probiotic-based  
40 strategy in controlling parasitic nematodes of socioeconomic importance.

41 **Importance**

42           Sequencing of the infected sheep 's stomach flora revealed potential probiotics  
43 that could control *H. contortus* infection, and further genetically engineered recombinant  
44 probiotic spores expressing parasite protein, and validated their good immunogenicity in a  
45 mouse model. In the sheep infection model, the recombinant probiotics have proven to be  
46 effective against parasite infections.

## 47 **Introduction**

48 *Haemonchus contortus* is one of the most economically important parasites  
49 causing haemonchosis in small ruminants around the world [1]. Haemonchosis may lead to  
50 anemia, weakness and even death of host animals prior to parasite's pre-patent period [2,  
51 3]. Anthelmintics have been the mainstay to control *H. contortus* infection. However,  
52 resistant *H. contortus* strains to widely used anthelmintics such as ivermectin are prevalent  
53 in many geographic regions [4]. Developing new prevention strategies against  
54 haemonchosis are challenging, although some progress has been made in addressing the  
55 mechanisms of *H. contortus* resistance [5]. Besides, residues of anthelmintics in meat, milk  
56 and other products impact human health and is a great public concern [6].

57 Probiotics are known to improve human and animal health. In particular, probiotics  
58 from food sources are thought to reduce intestinal infections by pathogens [7]. A previous  
59 study showed that *Bacillus subtilis* inhibited the colonization of *Staphylococcus aureus* by  
60 affecting its Agr quorum sensing system [7]. Bacterial spores can withstand extreme  
61 adverse environments with long-term survival rate. Therefore, the *Bacillus* spp. are  
62 considered as suitable probiotic candidates. In addition, *B. subtilis* spores have adjuvant  
63 effects [8, 9]. *B. subtilis* has recently been classified as the novel food probiotics for human  
64 and animal consumption and is widely used as oral vaccine vehicle [10], such as delivery of  
65 heterologous antigens to gastrointestinal tract as bioactive molecules [11, 12]. A recent  
66 study showed that CotC, a major component of the *B. subtilis* spore coat, was able to carry  
67 *Clonorchis sinensis* cysteine protease on the spore surface [13]. Recombinant *B. subtilis*  
68 spores expressing a tegumental protein was shown to provide protection against *C. sinensis*  
69 infection in a rat model [14].

70 HcGAPDH, an important component of *H. contortus* excretory/secretory products,  
71 is a glycolytic enzyme [15, 16]. In many organisms, GAPDHs are shown to have additional  
72 functions other than their enzymatic activity in glycolysis. It was shown that a recombinant  
73 HcGAPDH DNA vaccine can protect the recipient sheep from *H. contortus* infection by  
74 inducing effective host immune responses [17]. However, this DNA vaccine has not been  
75 applied in clinical practice, likely due to its limited commercial availability [18]. Given the  
76 prevalent anti-drug resistant for *H. contortus*, a better immune protection strategy against  
77 haemonchosis is still needed. The purposes of this study were to develop an oral vaccine  
78 using recombinant *B. subtilis* spores expressing CotB-HcGAPDH fusion protein and to  
79 investigate its underlying mechanisms. Overall, our data demonstrated a recombinant  
80 spore-based strategy as an alternative to anthelmintics.

## 81 **Results**

### 82 **Relative abundance of Bacillales negatively correlated with *H. contortus* infection**

83 To investigate the effect of microbiota on *H. contortus* infection, we analyzed  
84 abomasal microbiota of *H. contortus*-infected sheep using 16S ribosomal RNA (rRNA) gene  
85 sequencing. In the control sheep without *H. contortus* infection, the abomasal microbiota  
86 were dominated by the following bacterial class: Alteromonadales (35.5%),  
87 Pseudomonadales (29.5%), Bacteroidales (10.4%), Clostridiales (9.8%), Flavobacteriales  
88 (3.4%), Enterobacteriales (1.9%), Bacillales (1.3%), Aeromonadales (1.0%) (Fig 1a). *H.*  
89 *contortus* infection induced significant changes in microbial abundance including those of  
90 Alteromonadales, Pseudomonadales, Sphingobacteriales, Enterobacteriales, Bacillales, and  
91 Coriobacteriales, compared to the uninfected group (Fig 1a). Of particular interesting is the  
92 Bacillales group that has the probiotic effects in relation to *H. contortus* infection. We found

93 that the relative abundance of Bacillales was significantly reduced after *H. contortus*  
94 infection (Fig 1b and 1c) ( $p < 0.005$ ). In addition, our linear effect size (LEfSe) analysis on  
95 the 16S rRNA sequences showed that Bacillales is the main contributor as a probiotic in the  
96 abomasal microbiota to protect sheep from *H. contortus* infection (Fig 1d). Together, these  
97 data showed that sheep with *H. contortus* infection have led to significant reduction of  
98 Bacillales in the microbiota of abomasum, suggesting a potential protective role of these  
99 probiotic bacteria against pathogen infection.

100 **Expression of CotB-HcGAPDH on the surface did not affect the production or the**  
101 **structure of *B. subtilis* spores**

102 Based on the available data, we developed a protocol to generate recombinant  
103 spores expressing the fusion protein on the surface by joining the *B. subtilis* spore coat  
104 protein B (CotB) and the *H. contortus* GAPDH (HcGAPDH) protein (CotB-HcGAPDH or  
105 CotB-HcG) (Fig 2a). First, the full-length cDNA of *HcGAPDH* was cloned into the pET32a  
106 vector (pET32a-HcGAPDH), and the recombinant HcGAPDH protein was expressed and  
107 purified (Fig 2b and 2c). The purified protein was then used to generate polyclonal  
108 antibodies. Second, the *HcGAPDH* and *CotB* genes were fused and cloned into the  
109 pDG364 vector (pDG364-*CotB-HcGAPDH*). The fusion protein CotB-HcGAPDH (CotB-HcG)  
110 was expressed in *B. subtilis* spores (rBS<sup>CotB-HcG</sup>) (Fig 2d and Fig 2e).

111 To verify that the recombinant fusion protein was expressed on the surface of *B.*  
112 *subtilis* spores, we have performed the immunofluorescence (IF) using the polyclonal  
113 antibodies to HcGAPDH on the bacterial spores induced in Difco sporulation medium  
114 (DSM). CotB-HcGAPDH in rBS<sup>CotB-HcG</sup> started to appear on the spore coat after 24 h of  
115 induction and increased steadily between 24 h and 72 h (Fig 3a). Flow cytometry (FCM)

116 assay further confirmed that 86.01% of the rBS<sup>CotB-HcG</sup> spores expressed CotB-HcGAPDH  
117 72 h after induction (Fig 3b). There was no difference in production and germination of  
118 spores between the wild-type and the rBS<sup>CotB-HcG</sup> strains (Fig 3c). To determine whether  
119 expression of CotB-HcGAPDH affected spore structure, rBS<sup>CotB-HcG</sup> spores were examined  
120 using scanning electron microscope (SEM) and transmission electron microscope (TEM).  
121 There was no change on coat folds of elliptical spore morphology between the wild-type and  
122 the rBS<sup>CotB-HcG</sup> strains (Fig 3d). In addition, TEM images revealed clear exine and intine  
123 structures of rBS<sup>CotB-HcG</sup> similar to the wild-type strain (Fig 3e). These results indicate that  
124 expression of CotB-HcGAPDH fusion protein did not change the production or the structure  
125 of *B. subtilis* spores.

#### 126 **Recombinant *B. subtilis* spores expressing CotB-HcGAPDH fusion protein stimulated** 127 **both humoral and cell-mediated immune responses in mice and sheep**

128 To test whether the recombinant *B. subtilis* spores have positive probiotic effects in  
129 promoting immune responses, mice were orally administrated with PBS (Ctrl), wild-type  
130 (WT) strain, rBS<sup>CotB</sup> or rBS<sup>CotB-HcG</sup> spores, and purified HcGAPDH protein, respectively (Fig  
131 4a). Lymphocytes prepared from the spleen samples from mice treated as above were  
132 cultured and stimulated with ConA, LPS or the purified HcGAPDH protein to examine the  
133 specific cell-mediated immune responses. Both ConA and LPS groups showed that rBS<sup>CotB-</sup>  
134 <sup>HcG</sup> administration showed higher levels of lymphocyte proliferation than the control group  
135 ( $p < 0.01$ ) (Fig 4b). The purified HcGAPDH protein also stimulated lymphocyte proliferation  
136 with statistical significance as compared with the control group ( $p < 0.05$ ). To investigate  
137 whether humoral immune responses were activated by the rBS<sup>CotB-HcG</sup> spores, we measured  
138 anti-HcGAPDH Immunoglobulin G (IgG) levels in the murine sera. We found that rBS<sup>CotB-HcG</sup>  
139 induced the highest antibody level ( $p < 0.005$  as compared with Ctrl) at week 3 (Fig 4c). The

140 purified HcGAPDH protein also induced higher level of specific antibody than the control  
141 group ( $p < 0.01$ ). No anti-HcGAPDH antibody was detected in the mice receiving PBS, the  
142 wild-type or the rBS<sup>CotB</sup> strain ( $p > 0.05$ ). The subtype IgG2a or IgG1 reflects whether the  
143 type of T cell immune response is dominated by Th1 or Th2, respectively [19]. To further  
144 determine the Th1/Th2 phenotype of the T cell immune response triggered by rBS<sup>CotB-HcG</sup>,  
145 we found that the anti-HcGAPDH IgG2a was 2.07 folds higher than the anti-HcGAPDH IgG1  
146 ( $p < 0.005$ ), indicating a Th1 dominated T cell immune response (Fig 4d). We also evaluated  
147 the levels of anti-HcGAPDH secretory IgA (sIgA) from intestinal epithelial cells and plasma  
148 cells, which could protect animals from pathogen infection by mucosal immunity. The results  
149 showed that anti-HcGAPDH sIgA was significantly induced in intestinal mucus of rBS<sup>CotB-HcG</sup>  
150 mice in comparison to that of Ctrl ( $p < 0.01$ ) (S1 Fig). Genes representing Th1 activation  
151 (IFN- $\gamma$ , IL-2, IL-12, and T-bet) and those of Th2 activation (IL-4, IL-6, IL-10, and GATA-3) in  
152 splenic lymphocytes were significantly induced by rBS<sup>CotB-HcG</sup> administration (Fig 4e),  
153 suggesting that rBS<sup>CotB-HcG</sup> stimulated mixed Th1/Th2 immune responses. Collectively,  
154 these data indicate that *B. subtilis* spores expressing the CotB-HcGAPDH fusion protein  
155 activated both humoral and cell-mediated immune responses in mice.

156 We next investigated the immune responses stimulated by the rBS<sup>CotB-HcG</sup> spores  
157 in sheep, one of the natural hosts of *H. contortus*. An in vivo experiment was carried out by  
158 gavage with PBS (control, Ctrl), *H. contortus* infection (Hc), wild-type (WT) strain or rBS<sup>CotB-</sup>  
159 <sup>HcG</sup> spores followed by *H. contortus* infection (Ctrl, Hc, Hc+WT and Hc+rBS<sup>CotB-HcG</sup>) (Fig 5a).  
160 Lymphocytes from the peripheral blood (PBLs) of sheep were isolated and cultured at day 7  
161 after infection, a time point when *H. contortus* crawls to the abomasum and develops to the  
162 blood-sucking L4 stage. These cells were then stimulated with ConA, LPS or the purified  
163 HcGAPDH protein. Consistent with the murine results, proliferation of PBLs from sheep



164 receiving Hc+rBS<sup>CotB-HcG</sup> in the presence of ConA or LPS was significantly higher than that  
165 from Hc group ( $p < 0.005$ ) (Fig 5b). The purified HcGAPDH protein also stimulated  
166 significant proliferation of PBLs from these sheep in comparison to that from control sheep  
167 ( $p < 0.005$ ). Administration of rBS<sup>CotB-HcG</sup> induced the anti-HcGAPDH IgG production  
168 ( $p < 0.005$ , compared with Ctrl) at week 2, and the level plateaued at week 4 and maintained  
169 until week 8 (Fig 5c). Meanwhile, Anti-HcGAPDH IgG was not detectable in the control  
170 sheep. Further, anti-HcGAPDH sIgA levels were significantly higher in intestinal mucus of  
171 Hc+rBS<sup>CotB-HcG</sup> sheep than that of the Hc sheep ( $p < 0.01$ ) (S1 Fig). We also found that  
172 genes representing Th1 activation (IFN- $\gamma$ , IL-2, IL-12, and TNF- $\alpha$ ) and those of Th2  
173 activation (IL-4 and TGF- $\beta$ ) in PBLs of Hc+rBS<sup>CotB-HcG</sup> sheep were highly activated (Fig 5d)  
174 even though expression of IL-6 and IL-10 did not change ( $p > 0.05$ ). Collectively, these data  
175 show that rBS<sup>CotB-HcG</sup> stimulated strong humoral and cell-mediated immune responses in  
176 both mice and sheep.

### 177 **CotB-HcGAPDH recombinant *B. subtilis* spores promoted relative abundance of** 178 **probiotic Bacilli in the abomasal microbiota in sheep**

179 To investigate whether administration of rBS<sup>CotB-HcG</sup> affected abomasal microbiota  
180 of sheep in concomitant with *H. contortus* infection, 16S rRNA gene was sequenced from  
181 the abomasal mucus samples collected from the sheep of different treatment groups. Bacilli  
182 accounted for only less than 0.1% in the abomasal microbiota of sheep with *H. contortus*  
183 infection, compared with 4% of the controls (Fig 6a), consistent with our earlier findings (Fig.  
184 1). Bacilli from Hc+rBS<sup>CotB-HcG</sup> sheep accounted for 3%, indicating that administration of  
185 rBS<sup>CotB-HcG</sup> could restore Bacilli depleted by *H. contortus* infection (Fig 6a). Community  
186 taxonomic system composition analysis of Firmicutes indicated that administration of  
187 rBS<sup>CotB-HcG</sup> increased the relative abundance of Lactobacillales (Fig 6b). Specifically, the Ctrl,

188 Hc, Hc+WT and Hc+rBS<sup>CotB-HcG</sup> had a ratio of Lactobacillales abundance of 19.6%, 0.1%,  
189 3.9% and 76.8%, respectively, in taxonomic composition of Firmicutes (Fig 6c). These  
190 results indicate that administration of rBS<sup>CotB-HcG</sup> spores improved the composition of the  
191 microbiota by increasing the ratio of probiotic species in the abomasum of sheep infected  
192 with *H. contortus*.

### 193 **CotB-HcGAPDH recombinant *B. subtilis* spores protected sheep from *H. contortus*** 194 **infection**

195 To study the protective effect of rBS<sup>CotB-HcG</sup> on sheep against *H. contortus*  
196 infection, we measured the body weight and parasite loads of sheep. The average weight of  
197 the *H. contortus* infected sheep was only 49.5% of that of the non-infected control sheep,  
198 while sheep receiving rBS<sup>CotB-HcG</sup> at 10<sup>10</sup> or 10<sup>12</sup> CFU/animal followed by *H. contortus*  
199 infection could recover their body-weight back close to the controls. The wild-type *B. subtilis*  
200 spores also showed certain degree of protection against *H. contortus* infection, with 27.7%  
201 body weight gain compared to the infected sheep (Fig 7a). Next we determined parasite  
202 load by egg per gram feces (EPG) and adult worm counting. The sheep given 10<sup>10</sup> CFU of  
203 rBS<sup>CotB-HcG</sup> /animal followed by *H. contortus* infection had their EPG dropped to 71.5% (Fig  
204 7b). Their worm load also dropped to only 84.1% compared to the sheep infected with *H.*  
205 *contortus* (Fig 7c and Table 1). We also evaluated the infection by examining their  
206 abomasum. The surface of abomasum in the infected sheep was covered with worms and  
207 traces of parasite crawling. The numbers of worms and traces of parasite crawling in the  
208 Hc+rBS<sup>CotB-HcG</sup> sheep decreased compared with that of Hc group (Fig 7d). Further, the  
209 abomasum of infected sheep had intensive infiltration by mononuclear lymphocytes in  
210 mucosa, as compared with the un-infected sheep. No apparent infiltration was observed in  
211 Hc+rBS<sup>CotB-HcG</sup> sheep (Fig 7e). These data indicate that rBS<sup>CotB-HcG</sup> could offer effective

212 protection of sheep from *H. contortus* infection by promoting immune responses and  
213 improving microbiota (Fig 7f).

## 214 **Discussion**

215           The goals of the current study were to evaluate protective capacity of HcGAPDH  
216 engineered on the *B. subtilis* spore surface in sheep against infection by *H. contortus* and to  
217 elucidate the immunologic mechanisms for its protection. A recombinant *B. subtilis* strain  
218 rBS<sup>CotB-HcG</sup> was developed by expression of the *H. contortus* protein HcGAPDH fused to  
219 CotB on the spore coat. Such recombination and heterologous expression did not change  
220 production and structure of the spores. However, the rBS<sup>CotB-HcG</sup> played an important role in  
221 regulating abomasal microbiota favoring the host sheep particularly when they were infected  
222 by *H. contortus* with perturbed microbiota in the abomasum. The rBS<sup>CotB-HcG</sup> induced Th1-  
223 dominated immune responses in a mouse model, a mechanism through which it can offer  
224 effective protection for sheep from *H. contortus* infection, and thus alleviated damages  
225 triggered by parasitic infections.

226           Oral vaccination has great potential for field use as large amounts of particulate  
227 materials can be delivered with low risk of adverse side effects [20]. More importantly, the  
228 probiotic-based strategy of vaccination could minimize the use of anthelmintics, thus  
229 reducing the risk of anthelmintic residues in food and minimizing the development of drug  
230 resistance in parasites. It is well known that the strategy of antigen delivery affects the  
231 levels of immune responses [21]. Dominant antibodies in the mucus are the first line of host  
232 defense against various pathogens invading the mucosa including parasites [22-24], by  
233 inhibiting the motility and adherence of pathogens in the mucus [25]. Also, sIgA and IFN- $\gamma$   
234 have a strong bactericidal effect in the early stage of infection [26]. However, whether oral

235 administration is the best immunization strategy for *H. contortus* remains to be verified. Our  
236 data indicate that *B. subtilis* recombinant spores resisted the harsh conditions in the  
237 gastrointestinal tract and oral immunization with recombinant *B. subtilis* spores activated a  
238 strong mucosal immune response in the intestinal mucosa (S1 Fig).

239 Previous studies suggested delivery of the antigen via bacterial spores produced a  
240 Th1-biased cellular response, demonstrated by high levels of IgG2a [21]. Our results  
241 showed that murine splenic lymphocytes of rBS<sup>CotB-HcG</sup>-received mice expressed high levels  
242 of IL-2 and IFN- $\gamma$ , which suggested these cytokines might contribute to non-protective Th  
243 responses against *H. contortus*. In addition, IL-12 is a key cytokine that induces Th1 type  
244 immune response [27]. The transcription factor T-bet is a major regulator of Th1 cell  
245 polarization [28]. Significant up-regulation of IL-12 and T-bet gene expression induced by  
246 rBS<sup>CotB-HcG</sup> indicated that *B. subtilis* spores mainly elicited Th1-type immune responses.  
247 Interestingly, our results also indicate that the administration of rBS<sup>CotB-HcG</sup> in sheep induced  
248 the Th2 type immune responses besides Th1, as shown by up-regulation of cytokine IL-4  
249 and TGF- $\beta$ , implying that there may be mixed Th1/Th2 immune responses in sheep [29,  
250 30]. A possible explanation is that such mixed immune responses are jointly activated by  
251 spores and HcGAPDH antigenic protein. Alternatively, rBS<sup>CotB-HcG</sup> by proteolytic cleavage  
252 might release soluble antigens including HcGAPDH, following their uptake by antigen-  
253 presenting cells (APCs), which may lead to presentation of a major histocompatibility  
254 complex class II-restricted manner (MHC-II) for generation of Th2 type immune responses  
255 [31]. IL-4 is a signal cytokine for the Th2 type response and is mainly responsible for IgE  
256 isotype switching [32]. The immunosuppressive cytokine IL-10 is responsible for inhibition of  
257 Th2 immune responses [33, 34]. In mice, we found a slight upregulation of cytokines (IL-4,  
258 IL-10) and Th2 type transcription factors (GATA-3), suggesting that rBS<sup>CotB-HcG</sup> could induce

259 Th1/Th2 mixed immune responses. TGF- $\beta$  is a functionally multidimensional cytokine that  
260 manipulates various immune activities differentially in various cell types and potentially  
261 regulates a wide range of biological processes. In sheep, the mRNA levels of TGF- $\beta$  and IL-  
262 2 of lymphocytes in the peripheral blood were both increased. Some cytokines, particularly  
263 IFN- $\gamma$  and TGF- $\beta$ , have previously been proved to induce up-regulation of major  
264 histocompatibility complex class I-restricted manner (MHC-I) and MHC-II gene expression in  
265 different immune cells [35], which then stimulates production of antibodies and immune  
266 responses against parasitic pathogens [35]. Therefore, up-regulation of TGF- $\beta$  gene  
267 expression in the sheep receiving of rBS<sup>CotB-HcG</sup> suggested that spores presenting  
268 HcGAPDH protein may activate the host immune responses against parasitic infections by  
269 stimulating the MHC-I and MHC-II antigen presenting pathways. Our results are consistent  
270 with those in an early study using different recombinant spores [36].

271 Here we have shown an example of the combination of a probiotic strain and a  
272 subunit vaccine that could enhance protection against *H. contortus* infection. *H. contortus*  
273 infection leads to significant decrease in the abundance of Bacillales in the abomasal  
274 microbiota. In addition, previous studies have shown that *B. subtilis* spores possess  
275 adjuvant property due to the co. However, a correlation of *H. contortus* infection with  
276 specific changes at the species or genus level of bacteria was not found. One possible  
277 explanation is that sequencing-based approach is set up to detect high-order taxonomic  
278 shifts rather than specific differences at the species or the genus level, consistent with an  
279 earlier report [7]. We further investigated probiotics at the level of Bacillales for controlling *H.*  
280 *contortus* infection. In recent years, *Bacillus* spp. are widely used as probiotics in the  
281 livestock industry, with some European Union-approved products available in the market.  
282 The most notable one is BioPlus2B from Christian Hansen [37]. The probiotic *B. subtilis*

283 strain used as a carrier of passenger protein as vaccine has received attention because of  
284 its protective effects against various pathogens [20, 38-40]. Besides, previous studies have  
285 shown that *B. subtilis* spores possess adjuvant property due to the combination of antigens  
286 and spore surface [41, 42]. Many *Bacillus* strains are safe for sheep [43]. Therefore, they  
287 are likely suitable for use in sheep feeds. We have used these findings and attempted to  
288 establish a link between recombinant *B. subtilis* and prevention of *H. contortus* infection, by  
289 showing that the recombinant strain rBS<sup>CotB-HcG</sup> can offer significant protection against *H.*  
290 *contortus*.

291 Mechanistically, our working hypothesis (Fig 8) is that rBS<sup>CotB-HcG</sup> could activate T  
292 helper lymphocytes by APCs and stimulate up-regulation of IL-2 that might synergistically  
293 activate B lymphocytes to transform into plasma cells for generation of anti-HcGAPDH IgG  
294 antibodies. The spores could also stimulate the intestinal epithelial cells and plasma cells to  
295 produce anti-HcGAPDH sIgA, which would facilitate proliferation of eosinophils and up-  
296 regulation of TGF- $\beta$ , resulting in parasite clearance. More importantly, the HcGAPDH is a  
297 key protein in inhibition of host complement activation. *H. contortus* living inside the host  
298 releases HcGAPDH that is involved in evasion of the host immune system. Thus,  
299 administration of rBS<sup>CotB-HcG</sup> could induce anti-HcGAPDH IgG and sIgA to block immune  
300 evasion of *H. contortus*.

## 301 **Materials and Methods**

### 302 **Ethics approval**

303 Sheep (6 months of age; Huzhou, China) were maintained under helminth-free  
304 conditions in facilities at Zhejiang University. The procedures for animal maintenance and  
305 experiments were approved by Zhejiang University (permit no. ZJU20160239). All animal

306 experiments were performed in accordance with guidelines for the care and use of  
307 laboratory animals and the experiments were approved by Zhejiang University Experimental  
308 Animals Ethics Committee.

### 309 **Parasite**

310 *H. contortus* Zhejiang strain was kept at the Veterinary Parasitology Laboratory,  
311 Zhejiang University and maintained by serial passage in helminth-free sheep. Infective L3  
312 larvae (iL3s) were obtained by incubation of eggs for 14days at 28 °C.

### 313 **16S rRNA sequencing**

314 Six-month old female sheep were orally infected with 5, 000 *H. contortus* iL3s, and  
315 euthanized at 14, 31 or 62 days post infection (DPI). The control sheep received 1 ml of  
316 PBS by oral gavage and euthanized at 62 DPI. These sheep were housed in separated  
317 areas to avoid cross-contamination within the university facility under the same  
318 environmental conditions. Ten ml of abomasum fluids was collected from each sheep by  
319 squeezing the whole abomasum within 20 min of euthanasia. The abomasal fluids were  
320 centrifuged at 5, 000 ×g for 5 min at 4 °C. The supernatants were re-centrifuged at 12,000  
321 ×g for 10 min at 4 °C and the pellet of each sample was used for 16S rRNA gene  
322 sequencing of abomasal microbiota by the Illumina MiSeq® platform (Sangon Biotech,  
323 China). Raw sequence files have been deposited in the Sequence Read Archive database  
324 under the project SRP217048. [Python v1.2.2](#) was used to analyze both heatmap and  
325 community taxonomic system composition. [LEfSe v1.1.0](#) was used to analyze taxonomic  
326 cladogram. [Krona v2.6.1](#) was used for hierarchical analyses.

### 327 **Plasmid construction**



328           The 1,023 bp coding sequence (CDS) of HcGAPDH was amplified from the total  
329 cDNA of *H. contortus* by PCR using primers previously described [9]. PCR products were  
330 cloned into the pET-32a vector (Takara, China) via *Hind* III and *EcoR* I sites. The pET32a-  
331 HcGAPDH was sequenced (BioSune, China).

332           To generate a recombinant spore with a fusion protein of CotB-HcGAPDH, the  
333 genomic DNA of *B. subtilis* strain 168 was used as template to amplify the fragment of *CotB*  
334 gene (1,088 bp) containing the promoter sequence (263 bp) and *CotB* N-terminal partial  
335 CDS (825 bp) based on available sequences on NCBI (Reference Sequence:  
336 NC\_000964.3) using the primers as listed in S1 Table. PCR products were cloned into the  
337 pMD 18-T vector (Takara, China) at *Bam*HI and *Hind* III sites. The *CotB-HcGAPDH* was  
338 amplified by PCR with primers (S1 Table). The fused fragment of *CotB-HcGAPDH* was  
339 subcloned into *E. coli-B. subtilis* shuttle vector pDG364 (Miaolingbio, China) at *Bam*HI and  
340 *EcoR* I sites, resulting in pDG364-CotB-HcGAPDH plasmid. The control plasmid pDG364-  
341 *CotB* was constructed by cloning *CotB* gene directly into pDG364 vector. All the plasmids  
342 were confirmed by sequencing (BioSune, China).

### 343 **Expression of recombinant proteins**

344           The recombinant vector pET32a-HcGAPDH was transformed into *E. coli*. BL21  
345 strain. The transformants were cultured at 37 °C until the OD<sub>600nm</sub> value reached  
346 approximately 0.6 and were then induced with 0.5 mM isopropyl β-D-1-  
347 thiogalactopyranoside (IPTG) at 37 °C. The pellets were collected by centrifugation at  
348 8,000 ×g for 10 min, resuspended in buffer (0.01% digitonin, 10 mM Pipes, pH 6.8, 300  
349 mM sucrose, 100 mM NaCl, 3 mM MgCl<sub>2</sub>, and 5 mM EDTA) with proteinase inhibitors, and  
350 processed by sonication. The soluble His-tagged protein was purified using a HisTrap



351 column (GE Healthcare Life Sciences, USA). Purity of the eluted protein was checked by  
352 SDS-PAGE gel staining with Coomassie Blue. The anti-HcGAPDH rabbit polyclonal  
353 antibodies (rAb) were prepared according to the previous method [15]. The purified protein  
354 HcGAPDH and anti-HcGAPDH rAb were stored at  $-80\text{ }^{\circ}\text{C}$ .

355 The pDG364-CotB-HcGAPDH plasmid was linearized by *Kpn* I and transformed  
356 into *B. subtilis* strain 168 by electroporation [44]. The fusion gene *CotB-HcGAPDH*  
357 substituted for amylase E (*amyE*) gene in the genome of *B. subtilis* by homologous  
358 recombination. *B. subtilis* spores were prepared in 4 L of DSM for sporulation of the  
359 recombinant *B. subtilis* strain rBS<sup>CotB-HcG</sup> or rBS<sup>CotB</sup> as previously described [21]. They were  
360 purified by treatment with 4 mg/ml lysozyme followed by washing under stringent conditions  
361 in 1 M NaCl and 1 M KCl with 1 mM PMSF. Spores were treated at  $68\text{ }^{\circ}\text{C}$  for 1 h in water to  
362 kill any residual sporangial cells. The final concentration of spores was set at  $1 \times 10^{12}$   
363 CFU/ml in PBS pH 7.4. The spores were kept at  $-80\text{ }^{\circ}\text{C}$  until use for animal experiments.

#### 364 **SDS-PAGE and Western blotting**

365 The transformed *B. subtilis* strain containing pDG364-CotB-HcGAPDH (rBS<sup>CotB-</sup>  
366 <sup>HcG</sup>) was cultured in LB medium with 25  $\mu\text{g}/\text{ml}$  chloramphenicol at  $37\text{ }^{\circ}\text{C}$ . Bacterial  
367 sporulation was achieved by incubating in DSM according to the exhaustion method [21].  
368 Spores were harvested and analyzed by SDS-PAGE to evaluate the presence of HcGAPDH  
369 protein. Moreover, spore coat proteins were extracted from spores at 48 h of bacterial  
370 incubation in DSM medium using SDS-DTT extraction buffer (0.5% SDS, 0.1 M DTT, 0.1 M  
371 NaCl) as previously described [44]. The extracted proteins were subjected to 12% SDS-  
372 PAGE and then transferred onto Polyvinylidene Fluoride (Sigma, Germany). The  
373 immobilized filter was blocked overnight at  $4\text{ }^{\circ}\text{C}$  in 5% skimmed milk in PBST (PBS with

374 0.05% (v/v) Tween-20). Anti-HcGAPDH rAb (1:1000 in PBST) was used to probe the  
375 membrane by incubating for 2 h at RT after five washes in PBST. Finally, the probed filter  
376 was incubated with HRP-conjugated goat anti-rabbit IgG (1:5000 in PBST) and visualized  
377 by ECL (Beyotime biotechnology, China).

### 378 **Immunofluorescence and flow cytometry assay**

379 Five ml of sporulation cultures at 24 h, 48 h or 72 h of incubation were harvested  
380 and processed as previously described [45]. Samples were blocked with 5% bovine serum  
381 albumin (BSA) for 2 h at 4 °C followed by incubation with anti-HcGAPDH rAb (1:2,000 in  
382 PBST) for 2 h at room temperature. Naive pre-immunized rabbit sera (1:2,000 in PBST) was  
383 used as negative control. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG  
384 (Invitrogen, 1:500 in PBST) was used as the secondary antibody. Samples were observed  
385 and photographed under fluorescent microscope (Olympus BX51, Japan).

386 A total of  $1 \times 10^5$  purified spores were washed in PBS for 3 times and incubated  
387 with anti-HcGAPDH rAb (1:500 in PBST) at 37 °C for 2 h. Naive rabbit sera (1:500 in PBST)  
388 was used as negative control. After 3 washes in PBS, the spores were incubated with FITC-  
389 conjugated goat anti-rabbit IgG (1:500 in PBST, Invitrogen) at 37 °C for 1 h. Spores were  
390 finally resuspended in 1 ml of PBS following 3 washes, and at least  $1 \times 10^4$  spores were  
391 examined by FC500 MPL flow cytometer (Beckman Coulter, USA). Expression of the CotB-  
392 HcGAPDH fusion protein was analyzed using FlowJo software (Tree Star, USA).

### 393 **Analysis of the production and the structure of recombinant spores**

394 The purified spores of the wild-type strain and the recombinant strain rBS<sup>CotB-HcG</sup>  
395 were collected, fixed in 3% glutaraldehyde overnight at 4 °C followed by dehydration in  
396 gradient ethanol of 50%, 70%, 90% and 100%. After subsequent critical point drying and

397 sputter coating, they were processed and photographed under a scanning electron  
398 microscope SU-70 (Hitachi, Japan). For transmission electron microscopy, the spores were  
399 fixed in glutaraldehyde overnight at 4 °C followed by incubation in 4% osmium tetroxide for  
400 2 h. Afterwards, they were dehydrated in gradient ethanol (50%, 70%, 90%, and 100%),  
401 embedded and the ultrathin sections were mounted on a 230 mesh copper mesh, stained  
402 with 1% uranyl acetate-lead citrate. The spores were observed and photographed under a  
403 transmission electron microscope H-9500 (Hitachi, Japan).

404 To investigate whether production of spores of the recombinant strain rBS<sup>CotB-HcG</sup>  
405 was different from that of the wild-type strain, both strains were inoculated 1 L of DSM  
406 medium, cultured at 37 °C with constant shaking at 140 r/min. The number of viable  
407 bacteria and spores were then quantified.

## 408 **Animal experiments**

409 Six-week old female BALB/c mice were purchased from the Zhejiang Academy of  
410 Medical Science (Hangzhou, China), raised in a sterilized room, and fed with sterilized food  
411 and water. By oral gavage, the mice were administrated 100 µl PBS (Ctrl) per mouse,  
412 spores of wild-type strain at  $1 \times 10^{10}$  CFU (WT), rBS<sup>CotB</sup> at  $1 \times 10^{10}$  CFU (rBS<sup>CotB</sup>), and  
413 rBS<sup>CotB-HcG</sup> at  $1 \times 10^6$ ,  $10^8$ , or  $10^{10}$  CFU per mouse (rBS<sup>CotB-HcG</sup>), respectively. The mice in  
414 Ctrl, WT and rBS<sup>CotB-HcG</sup> groups were administrated for three consecutive days, followed by  
415 two boosting, each for three consecutive days, at a one-week interval. Mice of the  
416 HcGAPDH group were subcutaneously immunized with 200 µg of purified HcGAPDH  
417 emulsified in the complete Freund's adjuvant, followed by two boostings with 100 µg  
418 HcGAPDH emulsified in the incomplete Freund's adjuvant at a one-week interval. All mice

419 were euthanized at week 5 after the last immunization. Lymphocytes were isolated from  
420 spleens and cultured for extraction of total RNA.

421 Six-month old female sheep were purchased from the Miemieyang Animal  
422 Husbandry Co., Ltd. (Huzhou, China). All sheep were housed indoor and provided with hays  
423 and whole corns as food and water *ad libitum*. Each sheep was, by oral gavage,  
424 administrated with 1 ml PBS as control (Ctrl), spores of the wild-type strain (WT) at  $1 \times 10^{12}$   
425 CFU per sheep (Hc+WT), and spores of rBS<sup>CotB-HcG</sup> at  $1 \times 10^8$ ,  $10^{10}$  or  $10^{12}$  CFU per sheep  
426 (Hc+rBS<sup>CotB-HcG</sup>), respectively. The sheep were challenged with 5,000 *H. contortus* iL3s one  
427 week after the oral gavage. Serum samples were collected from the jugular vein of each  
428 animal every two weeks. All sheep were sacrificed at two months post infection. PBLs were  
429 isolated at 5 DPI using a sheep peripheral blood lymphocyte separation kit (Sangon  
430 Biotech, China). Body weight gain of each sheep was recorded as the difference in body-  
431 weight (kg) between week 11 and week 0. EPG was assayed at 14 DPI according to the  
432 modified McMaster method [28]. The numbers of *H. contortus* adult worms from abomasum  
433 in sheep were counted after euthanasia at week 11.

#### 434 **Lymphocyte proliferation assay**

435 As described previously [46], murine PBLs were stimulated with LPS (5  $\mu$ g/ml,  
436 Sigma, Germany), ConA (10  $\mu$ g/ml, Sigma, Germany) or purified HcGAPDH protein (15  
437  $\mu$ g/ml). The cells were evaluated for proliferation by MTT Assay Kit (Sangon Biotech, China)  
438 according to manufacturer's instructions. Experiments with sheep PBLs were performed the  
439 same way as the murine lymphocytes except for the concentration of LPS, ConA and  
440 purified HcGAPDH protein at 10  $\mu$ g/ml, 15  $\mu$ g/ml and 25  $\mu$ g/ml, respectively.

#### 441 **qRT-PCR assay**

442 Total RNA was extracted from PBLs. The cDNA synthesized by qPCR RT Kit  
443 (TOYOBO, Japan) was subjected to quantitative real-time PCR (qPCR) to measure the  
444 mRNA levels of cytokines and transcription factors using a SYBR Green PCR Master Mix  
445 (Applied Biosystems, USA) on a StepOnePlus Real-Time PCR System (Applied  
446 Biosystems, USA). The primers specific for mouse or sheep TGF- $\beta$ , IFN- $\gamma$ , IL-2, IL-12, IL-4,  
447 IL-6, IL-10, T-bet, or GATA-3 gene were listed in S1 Table.

#### 448 **Determination of antibodies by ELISA**

449 Serum samples were collected from each mouse weekly after administration of the  
450 spores. The intestinal mucus samples were collected at week 5 according to a method  
451 previously described [31]. The levels of anti-HcGAPDH IgG, sIgA, IgG1 and IgG2a were  
452 measured by ELISA. Briefly, ELISA plates (Bethyl, USA) were coated with 1  $\mu$ g purified  
453 HcGAPDH protein diluted in the coating buffer (0.05 M carbonate-bicarbonate, pH 9.6)  
454 followed by incubation in 5% skimmed milk for 18 h at room temperature. After three  
455 washes in PBST, the plates were then incubated at 37 °C for 2 h in serum or mucus in  
456 1:400 dilution in PBST. Subsequently, HRP-conjugated goat anti-mouse IgG (1:5,000  
457 dilutions, Abcam, UK), goat anti-mouse IgA (1:5,000 dilutions, Abcam, UK), goat anti-mouse  
458 IgG1 or IgG2a (1:1,000 dilutions, Abcam, UK) were employed as the secondary antibodies.  
459 After 1 h of incubation the plates were washed again and 100  $\mu$ l substrate solution 3, 3', 5,  
460 5'-tetramethylbenzidine (TMB, BD biosciences, USA) was added. After 5 min of incubation  
461 in dark, the reaction was stopped by adding 50  $\mu$ l 2 M H<sub>2</sub>SO<sub>4</sub>, and plates were read 3 times  
462 at 450 nm in the model microplate ELISA reader (BIO-RAD, Japan). Negative controls  
463 (coated with naive sera) were included on each plate. The results were expressed as  
464 OD<sub>450nm</sub> values. Similar to the mice serum protocol described above, anti-HcGAPDH IgG  
465 and sIgA in sheep samples were analyzed by ELISA as well. The secondary antibody was

466 HRP-conjugated rabbit anti-sheep IgG and rabbit anti-sheep IgA (1:5,000 dilutions, Abcam,  
467 UK).

## 468 **HE staining**

469 The abomasum dissected from sheep were thin-sectioned and subjected to HE  
470 staining [30]. The tissue sections were observed under an optical microscope (Zeiss,  
471 Germany).

## 472 **Analysis of abomasal microbiota from sheep**

473 Relative abundance of abomasal microbiota in sheep of the Ctrl, Hc, Hc+WT,  
474 Hc+rBS<sup>CotB-HcG</sup> groups were analyzed by 16S rRNA gene sequencing. Sampling and  
475 sequencing process were consistent with the previous protocol [47].

## 476 **Statistical analysis**

477 Results were presented as mean  $\pm$  S. E. M. (standard error of the mean). Means of  
478 continuous variables were tested with two-tailed Student's *t* test. *P* value of <0.05 was  
479 considered statistically significant.

## 480 **Data availability**

481 All the data supporting the findings of this study are available within the article and  
482 its supplementary files.

## 483 **Conflict of Interest**

484 The authors declare that the research was conducted in the absence of any  
485 commercial or financial relationships that could be construed as a potential conflict of  
486 interest.

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656

657 **Table.**

658 **Table 1. Worm reduction rate and EPG reduction rate in sheep with different treatments.**

	Worm count			Egg count		
	Mean	SD	worm reduction	Mean	SD	EPG reduction
PBS	0	0	na	0	0	na
Hc	690.8	205.0	–	70350.0	17753.96	–
Hc+WT( $10^{12}$ )	609.8	93.9	11.8%	50300.0	12145.45	28.5%*
Hc+rBS <sup>CotB-HcG</sup> ( $10^8$ )	332.5	106.5	51.9%**	65366.7	14908.21	7.1%
Hc+rBS <sup>CotB-HcG</sup> ( $10^{10}$ )	109.7	33.6	84.1%***	20050.0	5528.38	71.5%***
Hc+rBS <sup>CotB-HcG</sup> ( $10^{12}$ )	140.8	52.5	79.6%***	32766.7	5481.85	53.4%**

659 EPG: eggs per gram; na: Not applicable; \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.005$ .

660 **S1 Table. Primer sequence**

Primer ID	Primer sequence 5'-3'
CotB-F	CG <b>GGATCC</b> CAGCAAGAGGAGAATGAAATATC
CotB-R	CC <b>AAGCTT</b> AAATTTACGTTTCCAGTGATAG
Hc-GAPDH-F	CC <b>AAGCTT</b> ATGGTAAAACCAAAGGTTGG
Hc-GAPDH-R	CG <b>GAATTC</b> TTAGGCCTTGCTTGCAATGTAG
S-β-actin R	GCGTAGAGGTCTTTGCGGATGT
S-IL-2 F	AACGCTACAGAATTGAAACATC
S-IL-2 R	GTTTCAGATCCCTGTAGTTCCA
S-IL-4 F	TGTTCTGTGAATGAAGCCAAGACGAGTA
S-IL-4 R	ACCCTCATAATAGTCTTTAGCCTTTCCA
S-IL-6 F	AAACGAGTGGGTAAAGAACGCAAAG
S-IL-6 R	GAGGAGGGAATGCCCAGGAACTA
S-IL-10-F	GAGGTGATGCCACAGGCTGAGAA
S-IL-10-R	CTCCACCGCCTTGCTCTTGTTTT
S-IL-12-F	CAGCAGAGGCTCCTCTGAC
S-IL-12-R	GTCTGGTTTGATGATGTCCCTG
S-IFN-γ F	TAATGCAAGTAACCCAGATGTA
S-IFN-γ R	GCGTAGAGGTCTTTGCGGATGT
S-TGF-β F	GGCAGGTCATCACCATCGGCAAT
S-TGF-β R	GCCGACGTGACAGTAGAGGTAATAGAG
S-TNF-α F	CCAGAGGGAAGAGCAGTCC
S-TNF-α R	GGCTACAACGTGGGCTACC
M-β-actin F	CATCCGTAAGACCTCTATGCCAAC
M-β-actin R	ATGGAGCCACCGATCCACA
M-IL-2 F	CCCAAGCAGGCCACAGAATTGAAA
M-IL-2 R	AGTCAAATCCAGAACATGCCGCAG
M-IL-12 F	GGAAGCACGGCAGCAGAATAAAT
M-IL-12 R	AACTTGAGGGAGAAGTAGGAATGG
M-TFN-g F	TCTTGAAAGACAATCAGGCCATCA
M-TFN-g R	GAATCAGCAGCGACTCCTTTTCC
M-IL-4 F	CAAACGTCCTCACAGCAACG
M-IL-4 R	CTTGGACTCATTTCATGGTGC
M-IL-6 F	ACAACCACGGCCTTCCCTACTT
M-IL-6 R	CACGATTTCCAGAGAACATGTG
M-IL-10 F	GCTCTTACTGACTGGCATGAG
M-IL-10 R	CGCAGCTCTAGGAGCATGTG
M-T-bet F	GATCATCACTAAGCAAGGACGGC
M-T-bet R	AGACCACATCCACAAACATCCTG
M-GATA-3 F	AGTCCTCATCTTTCACCTTCC
M-GATA-3 R	GGCACTCTTCTCATCTTGCCT

## 662 **Figure legend**

663 **Fig 1. Relative abundance of Bacillales was related with *H. contortus* infection in**  
664 **sheep.**

665 **a.** Heatmap of relative abundance of abomasal bacteria. Color breaks in heatmap are  
666 adjusted to show relative abundance at <0.3% (blue shades), 0.3-0.4% (white shades),  
667 and >0.4% (red shades). DPI, Day post infection. **b.** Community taxonomic system  
668 composition analysis of Firmicutes. Relative abundance of abomasal bacteria in each  
669 sample is shown by a colored pie chart. **c.** The taxonomic composition of of Firmicutes. The  
670 proportion of different color blocks indicates relative abundance of different species. **d.** The  
671 taxonomic cladogram obtained by the linear effect size (LEfSe) analysis of 16S sequences  
672 within groups. Different colors represent different groups, and different color nodes in the  
673 branches represent groups of microorganisms that play an important role in the  
674 corresponding group of colors (a, Myroides; b, Sphingobacteriaceae; c, Sphingobacteriales;  
675 d, Sphingobacteriia; e, Anaerolineaceae; f, Anaerolineales; g, Anaerolineae; h, Bacilli; i,  
676 Lachnospiracea\_incertae\_sedis; j, Lachnospiraceae; k, Pseudoflavonifractor; l,  
677 Ruminococcus; m, Bulleidia; n, Erysipelotrichales; o, Erysipelotrichia; p, Veillonellaceae; q,  
678 Psychrobacter).

679 **Fig 2. Recombinant *B. subtilis* spores expressing CotB-HcGAPDH on the surface.**

680 **a.** Schematic of genetic engineering to generate recombinant spores with CotB-HcGAPDH  
681 presenting on the surface (rBS<sup>CotB-HcG</sup>). **b.** Coomassie blue staining of the recombinant  
682 HcGAPDH protein. M, protein marker; HcGAPDH, recombinant GAPDH from *H. contortus*;  
683 P, pellet; S, supernatant; IPTG, Isopropyl  $\beta$ -D-Thiogalactoside. **c.** Western blotting of the  
684 recombinant HcGAPDH with anti-His antibody. Vector, empty pET32a control. **d.**



685 Coomassie blue staining of the CotB-HcGAPDH fusion protein in *B. subtilis*. H, purified  
686 HcGAPDH protein; S, supernatant; C, spore coat from rBS<sup>CotB-HcG</sup>; DSM, Difco sporulation  
687 medium. **e.** Western blotting of the CotB-HcGAPDH fusion protein with anti-HcGAPDH  
688 rabbit antibody.

689 **Fig 3. Expression of CotB-HcGAPDH fusion protein did not affect the structure and**  
690 **production of *B. subtilis* spores**

691 **a.** Immunofluorescence (IF) of CotB-HcGAPDH expressed on the surface of wild-type (WT)  
692 and rBS<sup>CotB-HcG</sup> spores. 24 h, 48 h and 72 h indicate different time points after spore  
693 induction by DSM. BF, bright field; IF, immunofluorescence. Scale bar = 1  $\mu$ m. **b.** Flow  
694 cytometry (FCM) analysis of CotB-HcGAPDH expression on the surface of WT and rBS<sup>CotB-</sup>  
695 <sup>HcG</sup> spores. FS, forward scatter; SS, side scatter. **c.** Production and germination analysis of  
696 WT and rBS<sup>CotB-HcG</sup> spores. **d.** Representative images of WT and rBS<sup>CotB-HcG</sup> spores by  
697 scanning electron microscope (SEM). Scale bar = 10  $\mu$ m (left), 1  $\mu$ m (middle and right). **e.**  
698 Representative images of WT and rBS<sup>CotB-HcG</sup> spores by transmission electron microscope  
699 (TEM). Scale bar = 200 nm (left), 100 nm (right).

700 **Fig 4. Recombinant *B. subtilis* spores expressing the CotB-HcGAPDH fusion protein**  
701 **induced both humoral and cell-mediated immune responses in mice.**

702 **a.** Schematic of the experimental protocol in mice. Six-week old female mice (n = 20 in each  
703 group) administrated with PBS (Ctrl), WT, rBS<sup>CotB</sup>, rBS<sup>CotB-HcG</sup> spores, or purified HcGAPDH  
704 protein at indicated dosages. Serum samples collected at indicated time points. **b.**  
705 Proliferation of splenic lymphocytes of mice (n = 6 in each group) measured by MTT assay.  
706 **c.** Anti-HcGAPDH IgG levels in sera of mice (n = 6 in each group) at different time points. **d.**  
707 IgG1 and IgG2a levels in murine sera at week 5 (n = 6 in each group). **e.** The mRNA levels



708 of cytokine and transcription factor genes in splenic lymphocytes from mice (n = 6 in each  
709 group) measured by qRT-PCR. The dosage of rBS<sup>CotB-HcG</sup> was  $1 \times 10^{10}$  CFU. \* $p < 0.05$ ,  
710 \*\* $p < 0.01$ , and \*\*\* $p < 0.005$ . All data are presented as means  $\pm$  S. E. M. (standard error of  
711 the mean). Three technical replicates from a single experiment were used.

712 **Fig 5. The CotB-HcGAPDH fusion protein expressing recombinant *B. subtilis* spores**  
713 **stimulated both humoral and cell-mediated immune responses in sheep.**

714 **a.** Schematic of the experimental protocol in sheep. Six-month old female sheep were fed  
715 by gavage with PBS (Ctrl), WT or rBS<sup>CotB-HcG</sup> spores at indicated dosages during the first 3  
716 weeks, followed by *H. contortus* infection (n = 6 per group). Serum samples were collected  
717 at indicated time points. **b.** Proliferation of PBLs measured by MTT assay at week 4 (n = 6  
718 in each group). **c.** Anti-HcGAPDH IgG levels in sera of sheep (n = 6 in each group). **d.** The  
719 mRNA levels of cytokine and transcription factor genes in peripheral blood lymphocytes  
720 (PBLs) of sheep (n = 6 in each group) measured by qRT-PCR at week 4. The dosage of  
721 rBS<sup>CotB-HcG</sup> was  $1 \times 10^{12}$  CFU. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.005$ . All data are presented  
722 as means  $\pm$  S. E. M. Three technical replicates from a single experiment were used.

723 **Fig 6. The CotB-HcGAPDH fusion protein expressing recombinant *B. subtilis* spores**  
724 **promoted relative abundance of probiotic Bacilli in the abomasal microbiota in**  
725 **sheep.**

726 **a.** Hierarchical analyses depicted in the form of a Krona plot. Six-month old female sheep  
727 orally gavaged with PBS (Ctrl), WT or rBS<sup>CotB-HcG</sup> spores in  $10^{12}$  CFU/animal, followed by *H.*  
728 *contortus* infection (n = 3 in each group in all data). **b.** Heatmap of relative abundance at the  
729 class level of abomasal bacteria in sheep. **c.** Community taxonomic system composition

730 analysis at the class level in sheep. **d.** Taxonomic composition of Firmicutes. The proportion  
731 of different color blocks indicates the relative abundance of different species.

732 **Fig 7. The CotB-HcGAPDH recombinant *B. subtilis* spores protected sheep from *H.***  
733 ***contortus* infection.**

734 **a.** Average weight gain of sheep (n = 50 in each group). All data in this figure were collected  
735 from sheep followed the protocol shown in figure 5. **b.** Number of eggs per gram (EPG) of  
736 feces from sheep (n = 6 in each group). **c.** Number of worms from abomasum of sheep (n =  
737 6 in each group). **d.** Representative pictures of abomasum in sheep (upper panel). Zoom-in  
738 images to show the *H. contortus* in abomasum in sheep (lower panel). **e.** HE staining of  
739 abomasum in sheep. All values in a, b, and c are presented as mean  $\pm$  S. E. M. \* $p < 0.05$ ,  
740 \*\* $p < 0.01$ , and \*\*\* $p < 0.005$ . **f.** Schematic of the protective effect of the CotB-HcGAPDH  
741 recombinant *B. subtilis* spores on *H. contortus* infection.

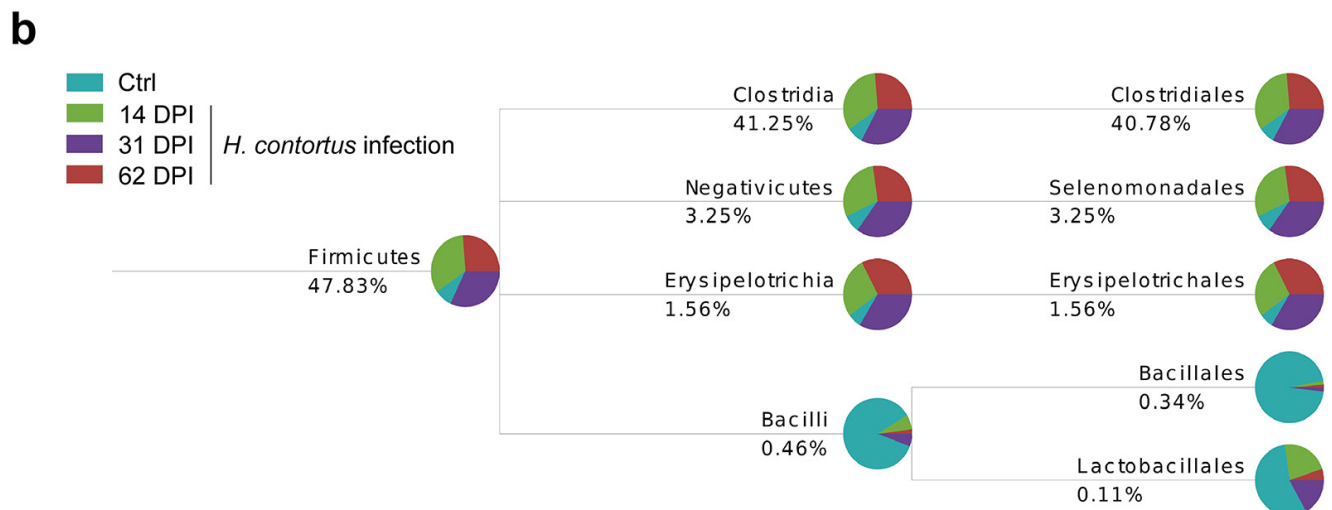
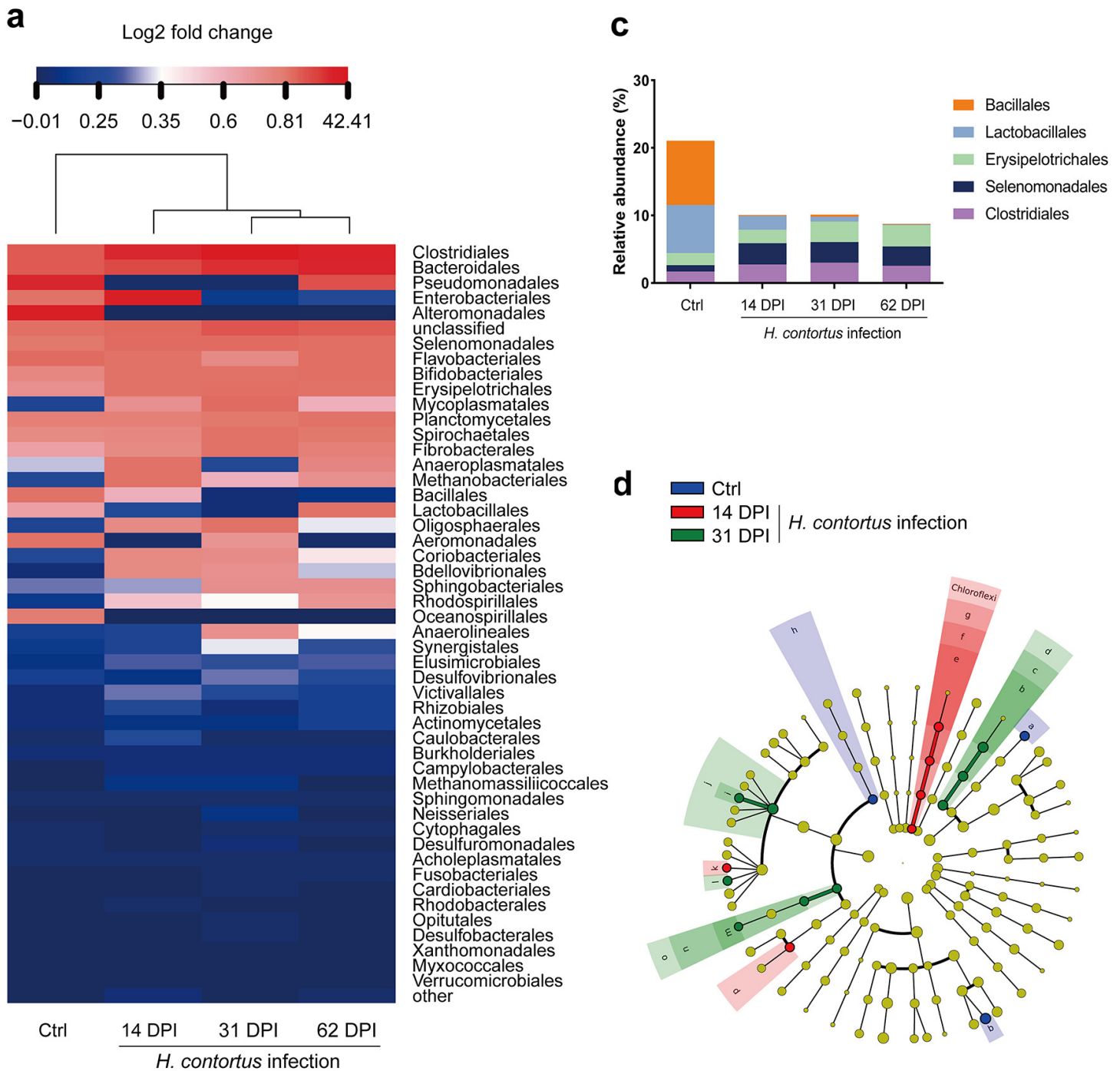
742 **Fig 8. The hypothetic scheme of the recombinant spores expressing the CotB-**  
743 **HcGAPDH fusion protein in protecting sheep from *H. contortus* infection by ADCC**  
744 **effect.**

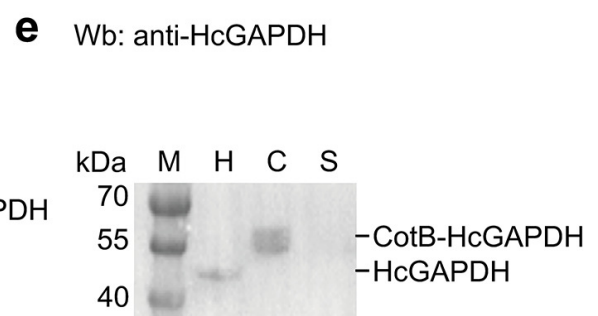
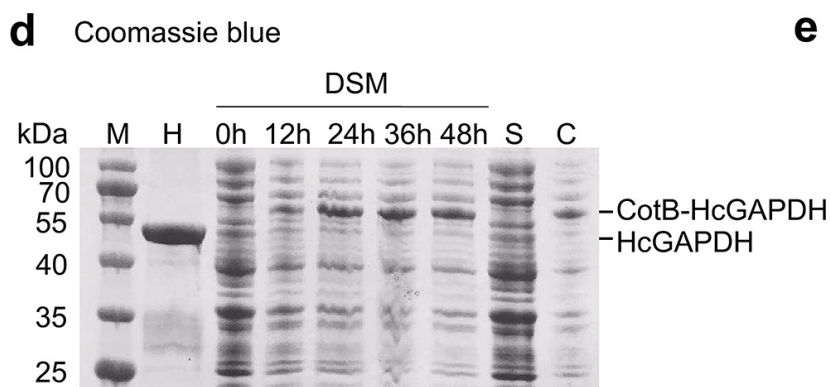
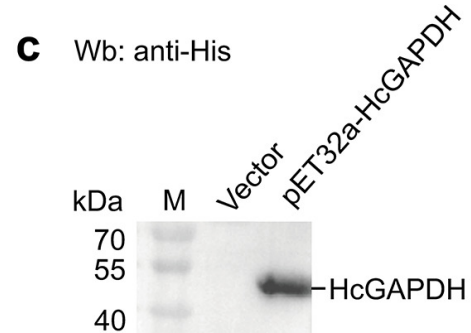
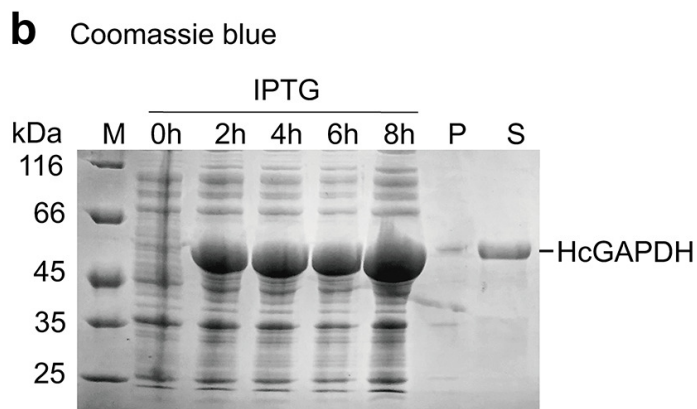
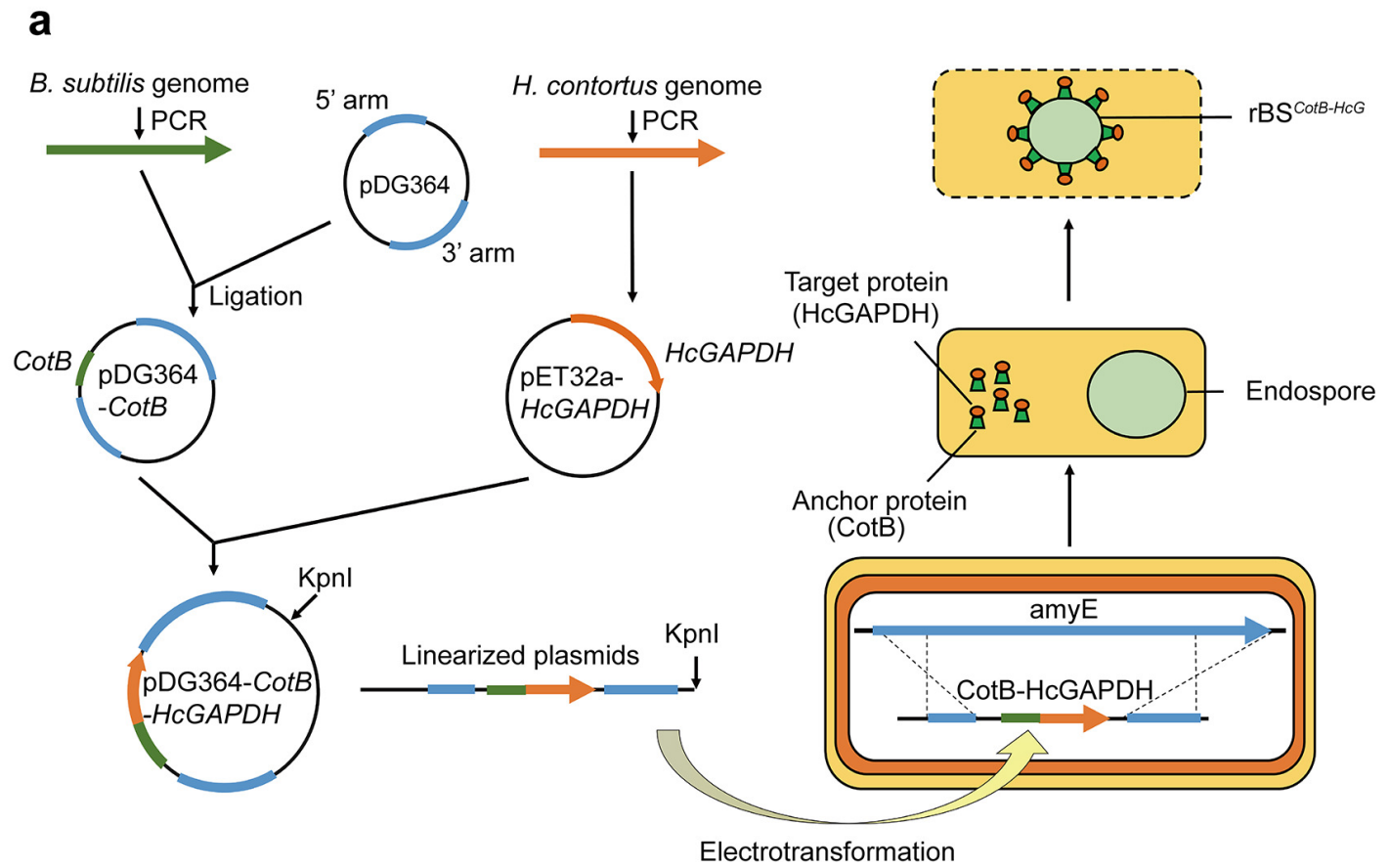
745 **a.** APC, antigen-presenting cells refer to a type of immune cells capable of ingesting,  
746 processing and processing antigens, and presenting the treated antigens to T and B  
747 lymphocytes. Th, helper T cell could help B cell produce antibodies. B, B lymphocyte. M $\phi$ ,  
748 macrophages. ADCC, antibody-dependent cell-mediated cytotoxicity.

## 749 **Supporting information**

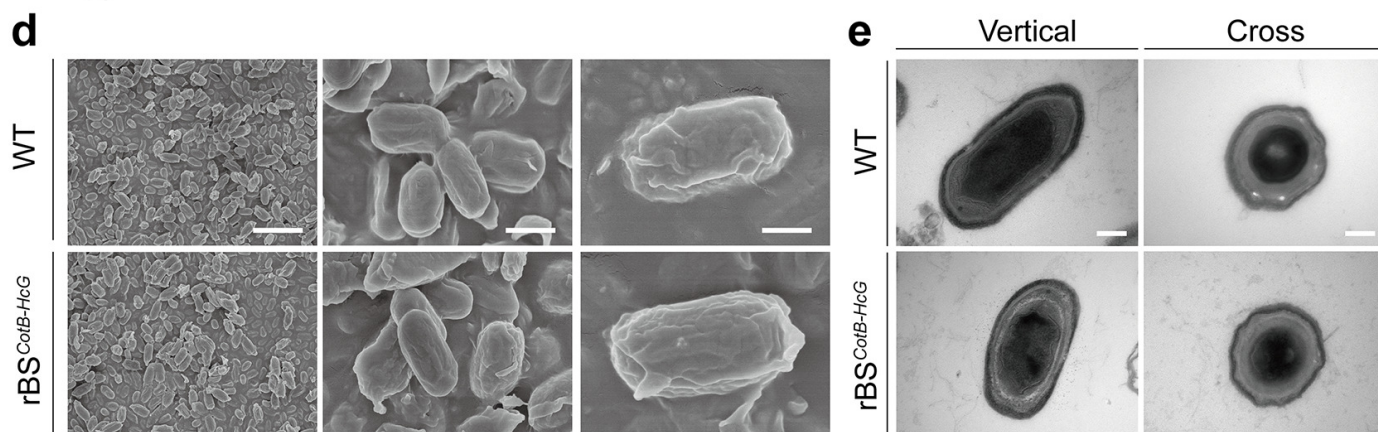
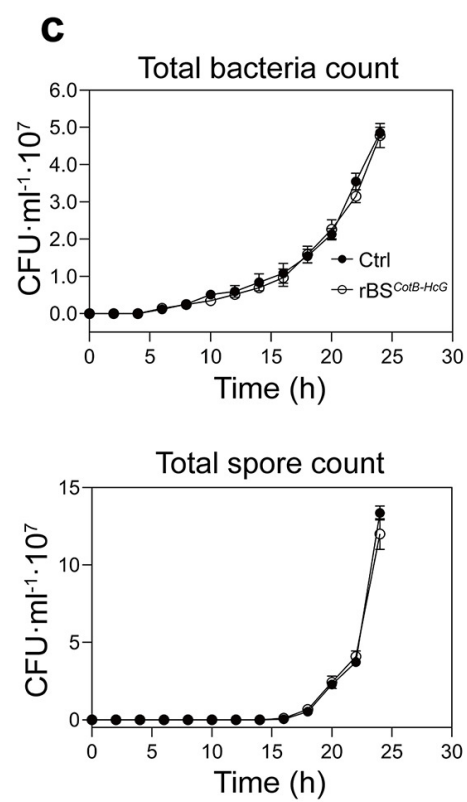
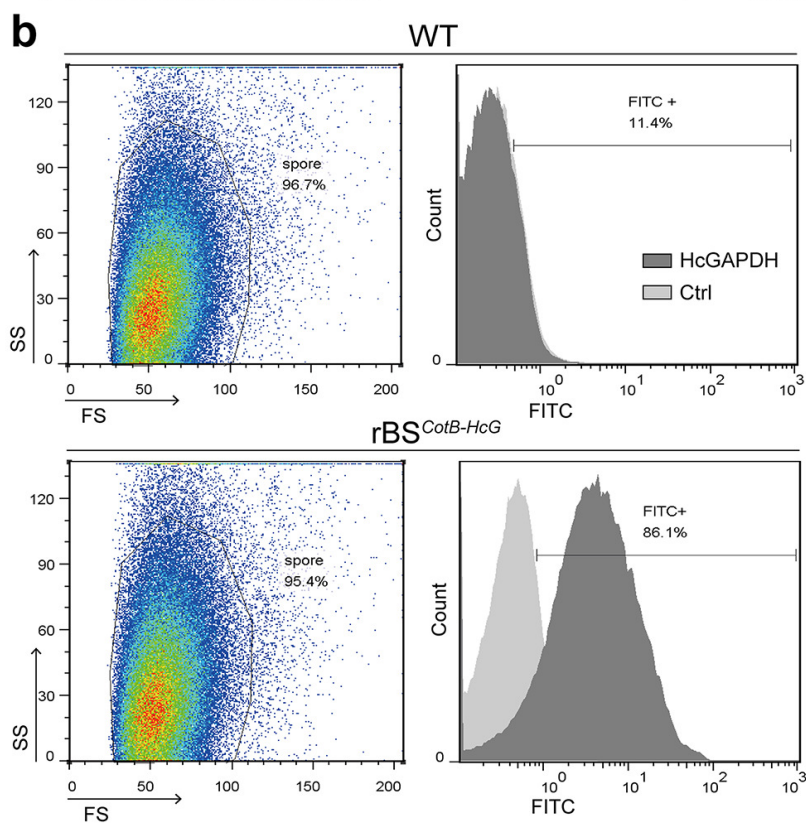
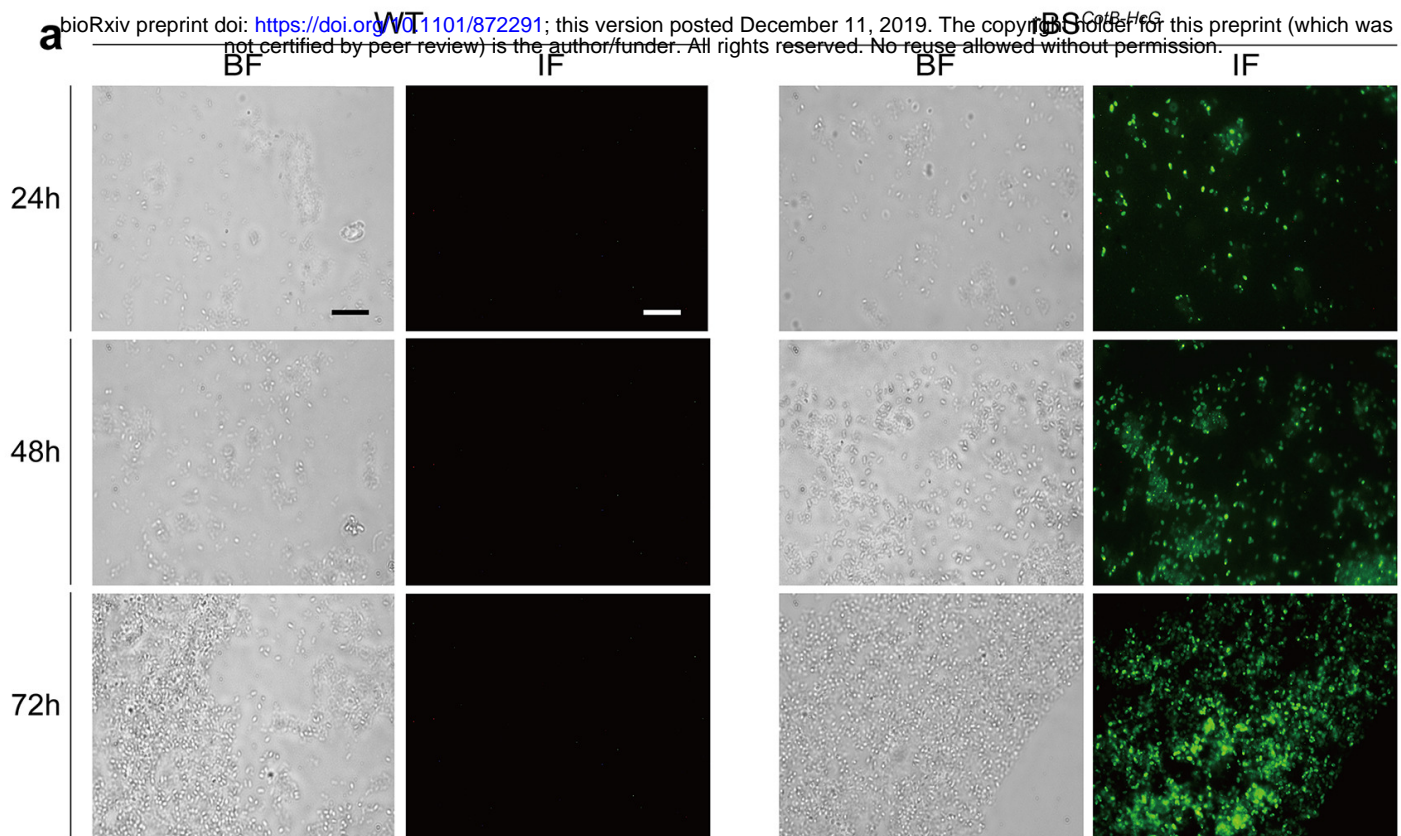
750 **S1 Fig. The CotB-HcGAPDH fusion protein expressing recombinant *B. subtilis* spores**  
751 **induced both humoral and cell-mediated immune responses in sheep.**

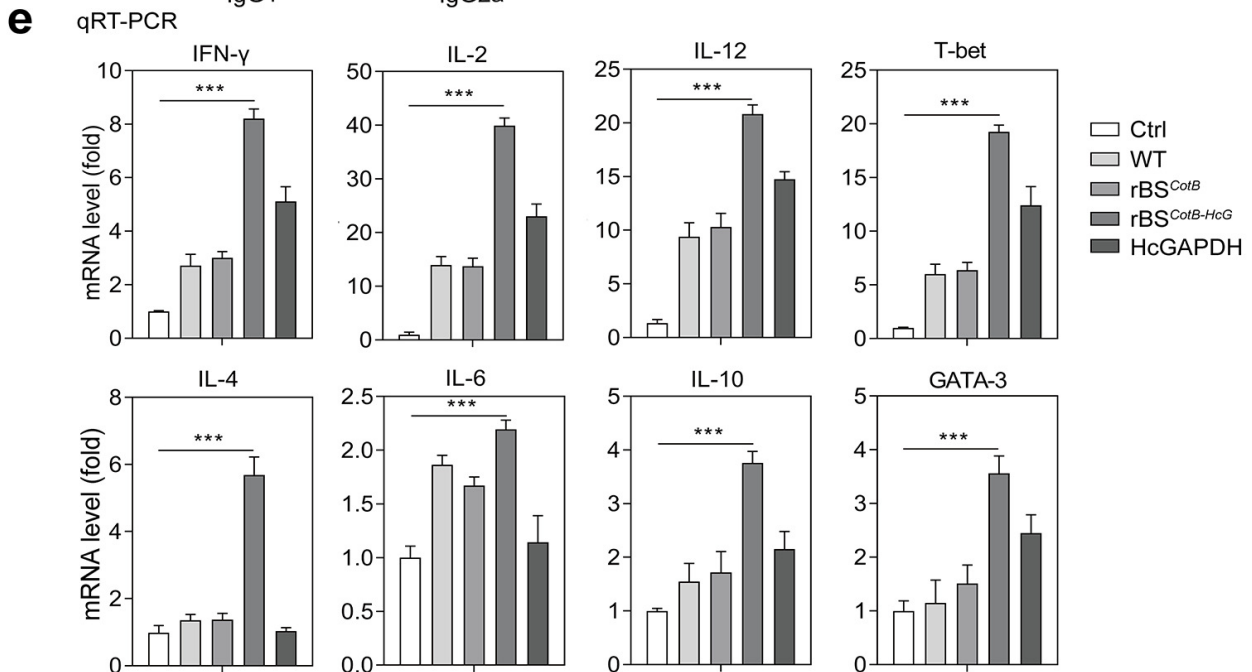
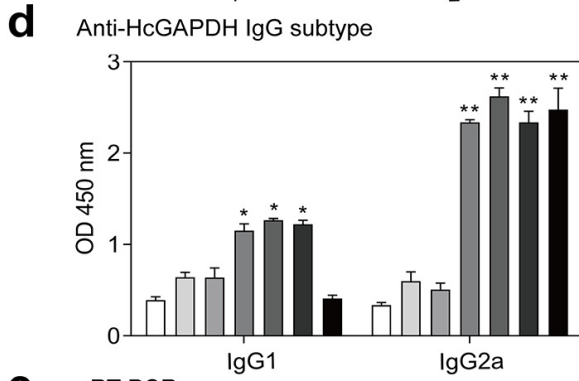
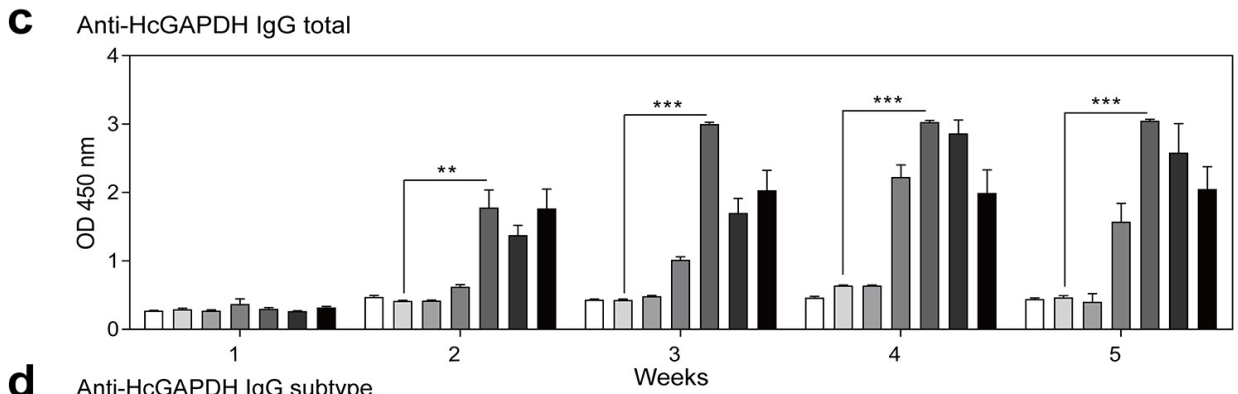
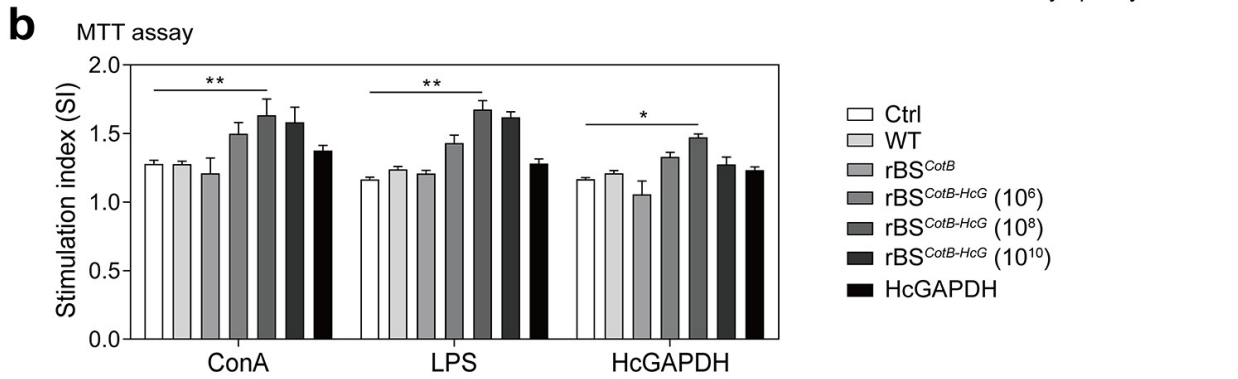
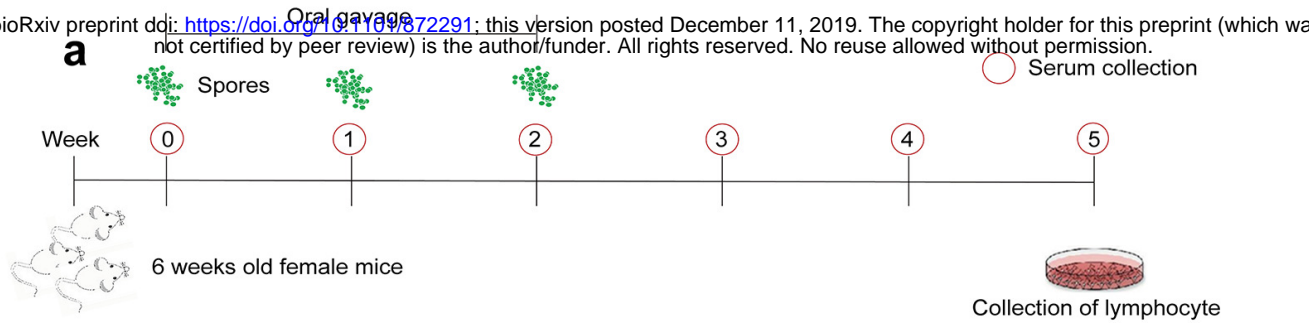
- 752 **a.** Anti-HcGAPDH sIgA levels in intestinal mucous samples of mice (n = 6 in each group). **b.**
- 753 Anti-HcGAPDH sIgA levels in intestinal mucous samples of sheep (n = 6 in each group).

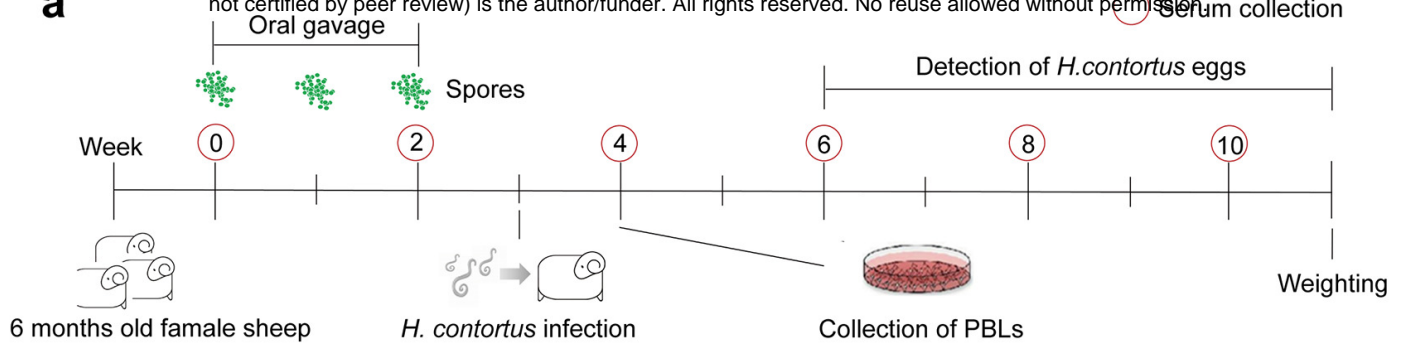




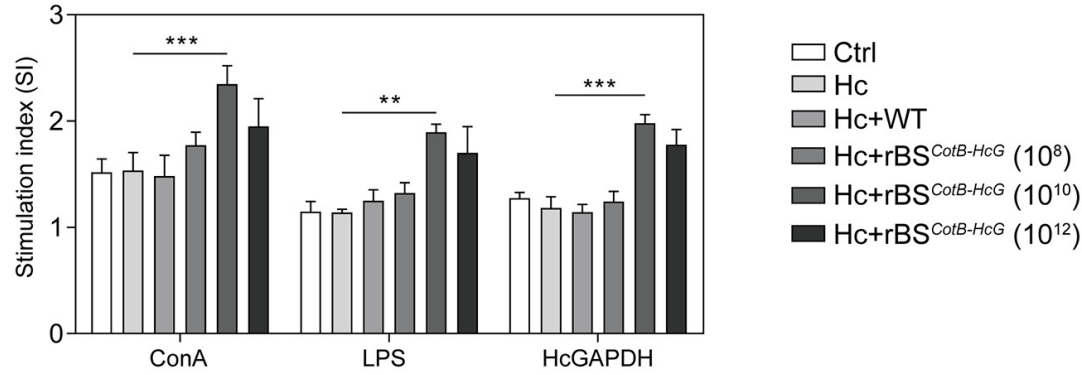




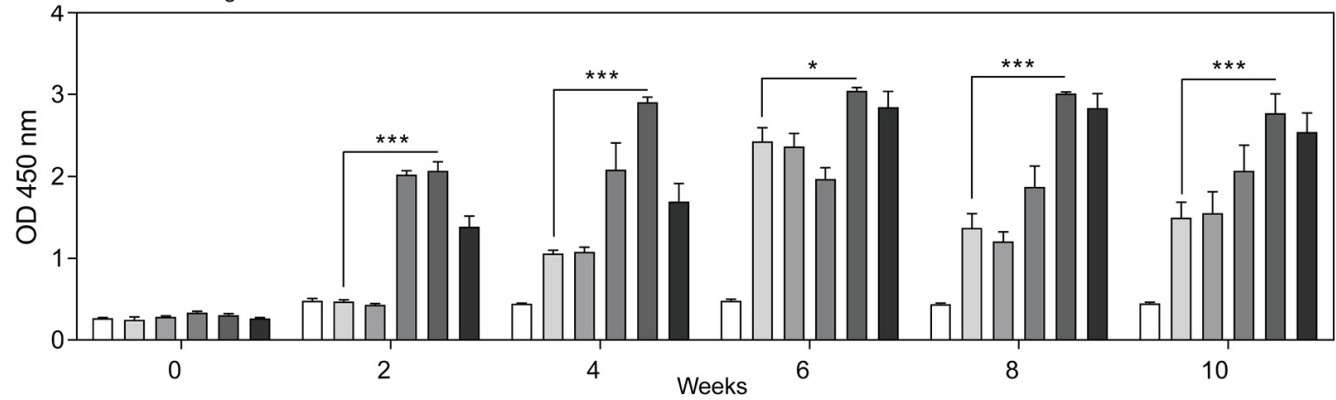




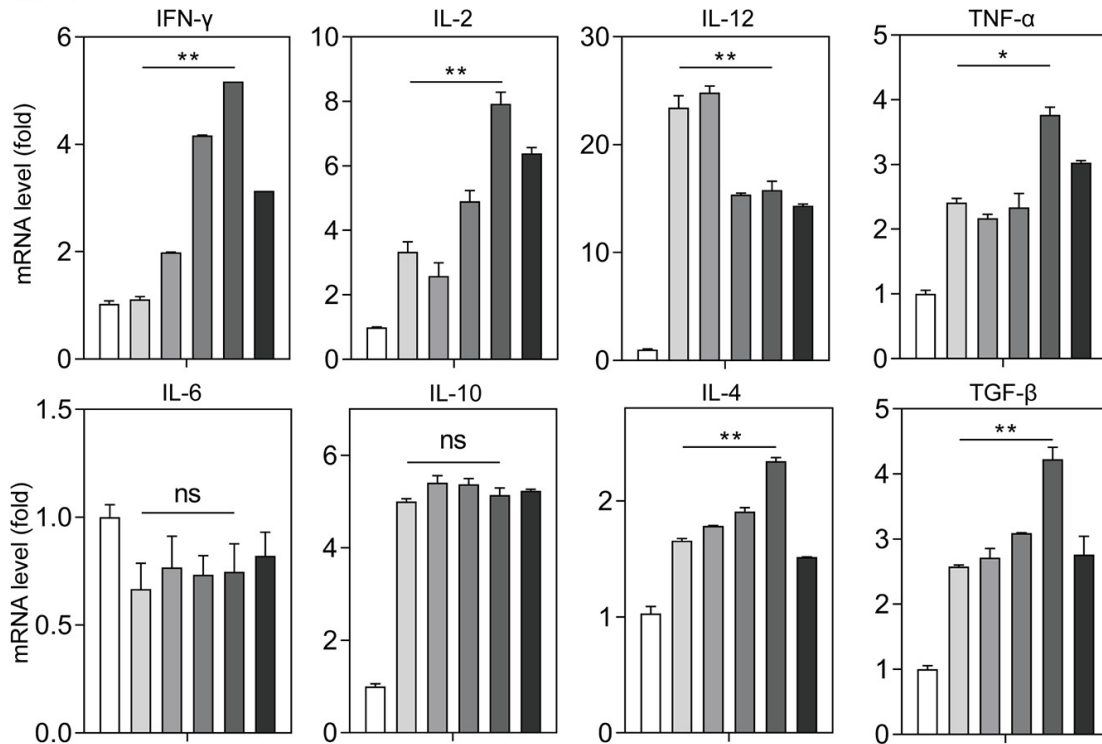
**b** MTT assay



**c** Anti- HcGAPDH IgG

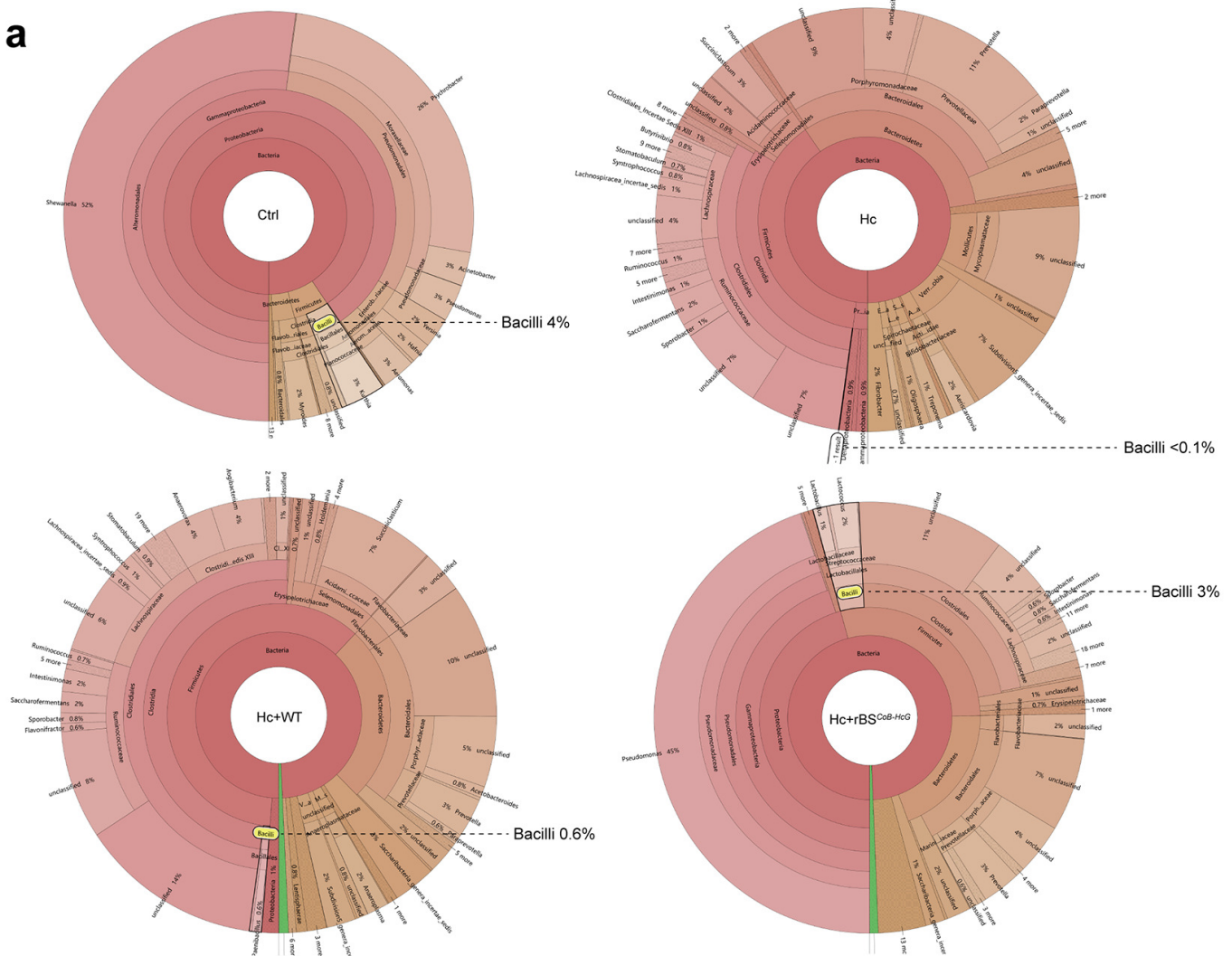


**d** qRT-PCR

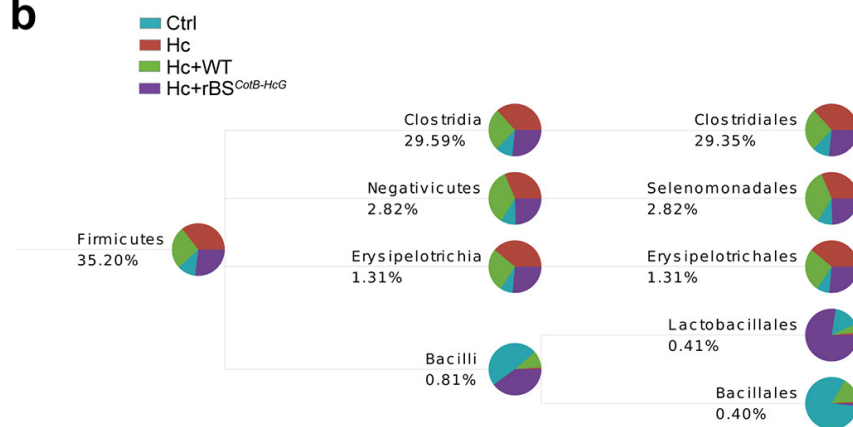




**a**



**b**



**c**

